Plant Molecular Biology Reporter **21:** 81–91, March 2003

Protocols

Conversion of an RAPD Marker to an STS Marker for Barley Variety Identification

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Abstract. Barley (*Hordeum vulgare* L.) variety identification is important to the malting and brewing industries. Because many new malting cultivars (varieties) are closely related, new and more effective identification techniques are needed. We report on a series of techniques used to convert an RAPD marker to a more stable STS marker that can identify barley Stander from Robust, an important distinction for the American malting and brewing industries. The techniques included DNA extraction, RAPD amplification, random cloning of all amplified fragments, selection of clones by insert size, DNA sequencing of select inserts, design of a barley-based primer pair, and detection of a single nucleotide polymorphism using restriction endonuclease *Alu* I. The barley-based primer pair was used to further sequence the RAPD fragment. Five single nucleotide polymorphisms between Robust and Stander exist, one of which was detected by electrophoresing DNA fragments differentially restricted by *Alu* I. The conversion technique was different from ones previously reported in that it did not require manual extraction of DNA fragments from a gel. This could be applied to other situations in which RAPD marker conversion would be desirable.

Key words: barley, RAPD marker conversion, SNP

Abbreviations: DTT, dithiothreitol; RAPD, random amplification of polymorphic DNA; TA, thymine-adenine overhangs; SNP, single nucleotide polymorphism; STS, sequence tagged site.

Introduction

The barley malting and brewing industries are in need of good methods to identify and distinguish malting barley (*Hordeum vulgare* L.) cultivars. Subtle differences in malting and brewing quality parameters of modern varieties often significantly affect the brewing process. Twenty-five years ago, the popular 6-rowed malting varieties grown in the north central United States could be distinguished solely by their seed morphologies, electrophoresis of their constitutive seed protein (Gebre et al., 1986; Heisel et al., 1986), or isoenzyme electrophoresis (Hoffman and Goates, 1990; Jones and Heisel, 1991). However, as barley

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breeders began to work within narrower gene pools, the need arose for more refined variety identification techniques.

Thus, barley geneticists have sought methods to detect DNA polymorphisms among elite barley varieties that share a narrow genetic base. RFLP has been extensively used for genetic mapping and to distinguish malting barley varieties (Saghai Maroof et al., 1994; Dahleen, 1997). However, it is time consuming and requires large amounts of clean restrictable DNA; thus, plants being analyzed must be grown for several weeks. Although RFLP is a superior mapping and genotyping technique, it is unsuitable for use in a quick variety identification test.

Some alternatives to RFLP include those based on the DNA polymerase chain reaction (PCR) procedure. PCR procedures are feasible for use in quick variety identification procedures. One PCR procedure uses short, randomly derived primers to amplify genomic DNA sequences and has been termed PCR-RAPD (Williams et al., 1990) or AP-PCR (Welsh and McClelland, 1990). Although this technique is widely used for genetic marker studies, it has not gained wide acceptance because of the lack of reproducibility among laboratories (Penner et al., 1993). RAPD is generally not used in practical variety identification because highly standardized procedures are required to use it successfully (Alex Kahler, personal communication). However, through optimizing PCR conditions and using *Taq* DNA polymerase, the Stoffel fragment, Hoffman and Bregitzer (1996) were able to reliably distinguish among a select group of closely related malting barley varieties in 2 laboratories.

Another PCR procedure, STS, uses primers of a known DNA sequence and has been used to differentiate barley varieties (Chee et al., 1993; Habernicht and Blake, 1999). It can be used as an alternative to RFLP for barley mapping (Tragoonrung et al., 1992). Initially, key variety distinctions were not reported in STS studies. These studies were later expanded, and STS polymorphisms between Stander and Robust were found (Habernicht and Blake, 1999).

We report a combination of methods to convert an RAPD marker to an STS marker and to test its ability to distinguish between Robust and Stander, an economically important comparison. This will provide additional markers for malting barley variety comparisons and in other applications where RAPD marker conversions are advantageous.

Materials and Methods

Plant material

Seeds of malting cultivars Morex, Robust, Stander, Excel, and experimental line M77 were obtained from the USDA-ARS Cereal Crops Research Unit Malt Quality Laboratory (Madison, Wisconsin, USA). Seeds of feed cultivar Steptoe were obtained from the USDA Small Grain and Potato Research Unit (Aberdeen, Idaho, USA). Plants were grown in 15-cm pots in a greenhouse during winter months. Fresh young leaves from 8-12 plants per cultivar in the 4-leaf stage were bulked for nucleic acid extraction.

Reagents and solutions

- 2 X CTAB DNA extraction buffer: 2% CTAB (w/v), 100 mM Tris-HCL (pH 8),

20 mM EDTA (pH 8), 1.4 M NaCl; autoclave and add 2% β-mercaptoethanol (v/v) immediately before use
- Chloroform-isoamylalcohol (IAA) (24:1 v/v)
- Wash buffer: 80% ethanol (95%) (v/v), 10 mM ammonium acetate
- 7.5 M ammonium acetate (pH 4.5)
- TAE buffer (pH 8.2): 40 mM Tris-acetate, 1 mM EDTA (pH 8)
- TE buffer (pH 8): 10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8)
- RAPD primers OPAB06 and OPAC09 (Qiagen Operon, Alameda, California, USA)
- PCR reagents (including polymerases) (Applied Biosystems, Foster City, California, USA)
- TA Cloning kit with pCR 2.1 vector and One-shot INVαF' chemically competent *Escherichia coli* (Invitrogen Life Technologies, Carlsbad, California, USA)
- Sequenase PCR Product Sequencing Kit #70170 (United States Biochemical/ Amersham Life Science, Cleveland, Ohio, USA)

Nucleic acid extraction[1]

- Place 1-2 g of fresh plant tissue in small mortar and grind to a fine powder in liquid nitrogen.
- Mix 20 mL of 2 X CTAB extraction buffer containing 40 uL BME with each ground sample (in a fume hood). Transfer to a 50-mL chloroform-resistant screw-cap centrifuge tube. Incubate at 65°C for 60 min.
- Cool solution to at least 50°C. Add 10 mL of 24:1 chloroform-isoamylalcohol.
- Invert tubes a few times. Centrifuge for 10 min at 1950 *g*.
- Carefully transfer upper aqueous phase to a new tube and repeat organic extraction.
- Add enough room temperature isopropanol to nearly fill tubes. Cap tightly.
- Turn tubes horizontally and gently tilt back and forth a few times.
- Spool nucleic acid precipitate with Pasteur pipette using a flame-formed hook.
- Place spooled nucleic acid in a 1-mL Eppendorf tube containing 0.5 mL of wash buffer for at least 15 min.
- Blot excess wash solution from the nucleic acid with tissue paper. Place in 0.5 mL TE buffer.
- Carefully remove Pasteur pipette after spooled pellet is partially dissolved. Close the tubes and allow the pellet to further dissolve overnight at room temperature.
- The next day, add 1 μL of 10 mg/mL heat-treated RNase, flick the tubes a few times, and incubate at room temperature for at least 2 h.
- After RNase treatment, estimate DNA concentration with light spectroscopy or fluorometry. Check the quality of extracted DNA with a 1% agarose gel.
- Prepare working solutions (10 ng of DNA per μL of sterile purified water) of each extraction.
- Store DNA stock and working solutions at 4°C in a refrigerator. Stock solutions may be stored for 1 y; working solutions should be remade every 2 wk.

Note:

1. Modified from Doyle and Doyle, 1987.

RAPD

- Make 3 μM primer stock solutions sufficient for 14 amplifications: 21 μL of 5 μM primer stock plus 14 μL of purified sterile water to make 3 μM primer working stocks.
- Place 2.5 μL of 10 ng/μL template DNA working stock solution and 2.5 μL of 3 μM primer into each of 14 PCR tubes.
- Prepare a master mix for 14 PCR reactions: 161 μL of sterile purified water, 35 μL Stoffel PCR buffer, 52.5 μL of 0.1 M magnesium chloride, 7 μL of each dNTP, and 3.5 μL *Taq* DNA polymerase, Stoffel fragment. Mix contents by lightly shaking. (In a 25 μL PCR mix, this results in a final concentration 0.3 μM primer, 1.5 mM magnesium chloride, 0.02 μM each dNTP, and 2.5 U *Taq* DNA polymerase, Stoffel fragment.)
- Add 20 μL of master mix to each PCR tube, mix by lightly flicking the tubes, and spin the tubes briefly in a microcentrifuge to gather the contents. If necessary, overlay the PCR reactions with 20 μL of mineral oil.
- Place the tubes into a PCR thermocycler programmed as follows: 2 min of initial denaturation at 94°C, followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 36°C, and 2 min of extension at 72°C. Follow with a single 7-min extension at 72°C.
- Electrophorese 12 μL of amplified products (about half of the reaction) plus 1.5 μL of PCR-loading buffer in 2% agarose gels covered with TAE buffer for 3 h at a constant 40 v/cm gel.
- Stain gel with 200 mL of ethidium bromide at a concentration of 0.5 μg/mL TAE solution for 30 min and rinse with distilled water.
- Illuminate rinsed gel with a 302-nm UV light and capture image digitally or on film such as Polaroid 55 or 57.

Cloning of RAPD fragments and insert size selection of clones

- Follow instructions provided with the TA Cloning kit with pCR 2.1 vector and One Shot INVαF' chemically competent *E. coli* (Invitrogen).
- Pick 28 (instead of 10 per instructions) white colonies from a plate yielding several well-spaced colonies and separately replate the colonies onto fresh LB plates. A flamed loop or a sterilized toothpick works well.
- Label and invert plates. Incubate at 37°C for at least 18 h.
- Make 2 μM primer stock solutions sufficient for 28 amplifications: 28 μL of 5 μM primer stock plus 42 μL of purified sterile water.
- Place 2.5 μL of each of the 2 primer working stocks into each of 28 PCR tubes.
- Prepare a "master mix" for 28 PCR reactions: 322 μL of sterile purified water, 70 μL PCR buffer, 42 μL of 0.1 M magnesium chloride, 28 μL of each dNTP, and 5.2 μL of *Taq* DNA polymerase. (In a 25 μL PCR mix, this results in a final concentration of 0.2 μM of each primer, 1.5 mM magnesium chloride, 0.02 μM of each dNTP, and 0.9 U *Taq* DNA polymerase.)
- Mix contents by gently flicking tubes. Spin 1-2 s in microcentrifuge.
- Add 20 μL of the master mix to each PCR tube containing the M13 primers. Transfer one colony from each restreaked LB plate and agitate with PCR solution of individual tubes, one colony per tube.

- Overlay the contents of the PCR tubes with 20 μL of mineral oil, if necessary.
- Subject to PCR as for RAPD but use an annealing temperature of 50°C.
- After PCR, electrophorese 5 μL of the amplified on 1% agarose gels and stain as described above. Estimate size of amplified fragments on the basis of a 100-bp standard.
- Select 1 or 2 clones that have inserts the same length of the band of interest plus any amplified flanking plasmid. With M13 primers, 180 bp of flanking pCR®2.1 plasmid DNA will be amplified.

PCR-primer initiated double-stranded DNA sequencing of size-selected inserts

- Follow instructions provided in the Sequenase PCR Product Sequencing Kit #70170 (United States Biochemical/Amersham Technologies).
- Following sequencing reactions. Heat DNA sequence samples to 75°C.
- Load 2 μL of each sample onto a 6% polyacrylamide sequencing gel that has been prerun at 50 w for 30 min.
- Electrophorese samples for 2 h at 50 w.
- Dry gel and expose to x-ray film for 18 h.
- Develop, dry, and read film. Approximately 200 bases can usually be read from each end.

Primer design and additional sequencing

- Use sequence information and primer design software to select 6 or 7 highly specific primer pairs free of dimer and hairpin structures and form stable duplexes with the DNA template.
- Test the newly designed primers in PCR reactions and 1% agarose gel electrophoresis.
- Use the same procedure as described for amplification with M13 primers, except make 4-μM primer solutions, place 1.75 μL of each of 2 paired barley primers in a PCR reaction tube, and add 2.5 μL of 10 ng/μL template DNA. (We used cultivar Morex DNA.)
- Use newly designed primers and target organism DNA as template (in our case, DNA of cultivars Robust and Stander) to generate variety-specific DNA sequence information with the Sequenase DNA sequencing kit as described above, except substitute the M13 primer pairs with the newly designed primer pairs.
- Compare sequence information for single nucleotide polymorphisms (SNPs) and check if any of the detected SNPs can be detected by means of restriction digest.
- Verify findings with PCR (as above) and agarose gel electrophoresis or PAGE.

Notes:

1. If fresh young tissue is used, nearly all nucleic acid extractions will result in "spoolable" precipitates. If such precipitates are not obtained, repeat the procedure or centrifuge again (see Doyle and Doyle [1988] for more details).

2. The number of PCR reactions to set up at a time varies, but multiples of 14 worked well for our 30-well gels (28 sample lanes and 2 DNA-size standard lanes).
3. We added 1 µL of PCR reaction in the ligation mix to approximate a 1:1 insert-to-vector ratio for optimal cloning efficiency, and we used 3 µL of pretreated PCR product in the annealing mixture for DNA sequencing. The amounts of amplified DNA used in the cloning procedure and in the annealing mixture for DNA sequencing may need to be adjusted to account for DNA insert size and/or DNA concentration.

Results and Discussion

RAPD amplification and electrophoresis

Using RAPD primers OPAB06 and OPAC09 gave the same results as previously reported (Hoffman and Bregitzer, 1996). Figures 1 and 2 show RAPD differences among an array of elite 6-rowed malting barley cultivars from a narrow genetic base (Table 1). The malting and brewing industries are interested in quick and precise methods to distinguish between malting barley cultivars such as Robust and Stander. As new cultivars are developed and released, other comparisons will become important.

Cloning and sequencing RAPD-generated DNA fragments

Two clones were identified with inserts of approximately 1130 bp. After subtracting the 180 bp of the amplified flanking vector sequence, these clones apparently contained the OPAC09-generated 950-bp discriminatory fragment. No clones were found to have inserts corresponding to the 1250-bp discriminatory RAPD fragment amplified by OPAC09 and the 1175-bp fragment amplified by OPAB06. The large fragments (>1100 bp) plus the additional 180-bp vector DNA may have been too large for efficient ligation. The TA Cloning kit instructions state that fragment size influences ligation efficiency. The relatively low amounts of amplification of the larger fragments (Figures 1 and 2) and small sample size may also have contributed to the lack of large fragment clones.

The terminal sequences of the chosen OPAC09 950 bp clone are listed in Table 2. Three suitable primer pairs that could be used to further sequence the 950-bp fragment were identified. One primer pair (#3 and 4, Table 2) effectively amplified a PCR product of approximately 800 bp (Figure 3). A partial DNA sequence of the 800-bp DNA fragment is given in Table 3. Direct cloning of all RAPD fragments is followed by size-selection of clones as long as fragments are not too large for efficient cloning. This method is an alternative to manually isolating DNA fragments from gel, in which single-fragment recovery is not always achieved and extracted DNA fragments must be cleaned prior to cloning.

Detection of SNPs and a restriction site polymorphism

DNA sequencing using Stander and Robust genomic DNAs as templates revealed 5 putative SNPs in the DNA of these 2 cultivars (Table 3). One SNP caused an

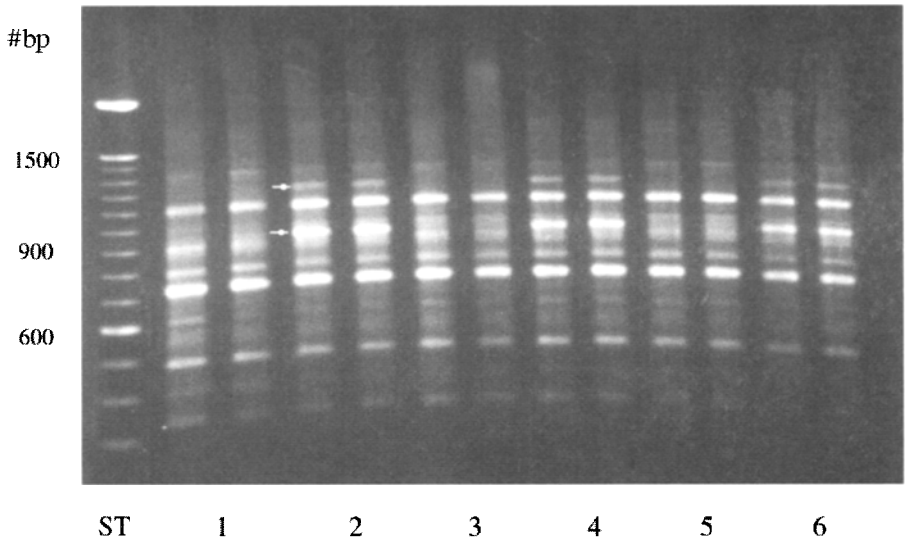

Figure 1. Agarose gel separation of DNA fragments resulting from the amplification of DNA of various barleys with RAPD primer OPAC09. The lane marked ST was loaded with 0.5 ng of a 100-bp DNA standard. Lane pair 1 was loaded with amplified DNA of cultivar Steptoe; lane pair 2, Morex; lane pair 3, Robust; lane pair 4, Stander; lane pair 5, Excel; lane pair 6, experimental line M77. PCR fragments were cloned into TA plasmid, and clones were screened for plasmids containing either the 950-bp or 1250-bp fragment (indicated with an arrow).

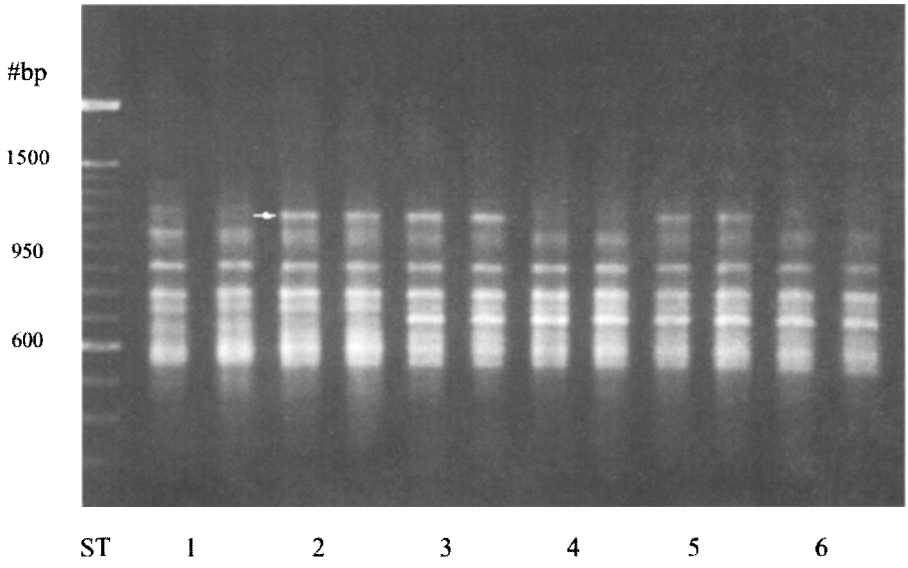

Figure 2. As in Figure 1, except that RAPD primer OPAB06 was used and screening was conducted for clones containing the 1175-bp fragment (indicated with an arrow).

Table 1. Six-rowed malting barley varieties and one experimental line studied for RAPD DNA polymorphisms, including pedigrees and the year of the *Crop Science* registration notice.

Variety/line	Pedigree	Year
Morex	Cree/Bonanaza	1979
Robust	Morex/Manker	1983
Stander	Excel//Robust/Bumper	1993
Excel	Cree/Bonanaza//Manker/3/2*Robust	1991
M77	Stander/Excel	——

Table 2. The DNA sequence of the ends of TA Clone that correspond to a discriminatory 950-bp RAPD fragment that was amplified by primer OPAC09. The sequences in bold indicate the designed primer pair #3 and 4 that was used to internally sequence the RAPD fragment and for the STS analysis. The dotted lines indicate a stretch of unreadable sequences.

5'

AGAGCGTACCAAAAAAGGAGGGGAAACAAGAACTTTAGGACGTATGCGCCATAAAGCGAGTAGCC

3'...TCCTGCATACGCGGTATTTC...5'

#3) 5' – **AGGACGTATGCGCCATAAAG** - 3'>>

CCGAAGTACAACCCATGGTCGGCATGTCGATCACCTATGACCGCATGGATCACCCAACCTGTATT

3' 5'

CGGCAT.....CCATGTATTTGGTTAAACCGAGCTATGGTCATCACATGTCCTCGACAAATCCAGGTAC

#4) <<3'- **TACAGGAGCTGTTTAGGTCC** -5'

CATAAAGGACCAACTCAGCGTCAAGCTACACATACCTCGGCGACTTCTCAGGACCACCTGTCTTAA

3'

AGGTACGCTCT

Alu I restriction site difference. Cutting the 800-bp PCR product with *Alu* I followed by electrophoresis in a 6% PAGE verified the presence of an *Alu* I–specific SNP restriction site difference between the DNA of Robust and Stander (Figure 3). This procedure provides a way to directly target STS-detectable SNPs and precludes testing several endonucleases until a desired polymorphism is found. PCR-directed sequencing of genomic DNA templates eliminates the need for cloning each fragment to be sequenced.

Conclusion

This paper reports a series of methods to readily convert an RAPD marker to STS marker that can be used for identifying specific barley varieties. The technique

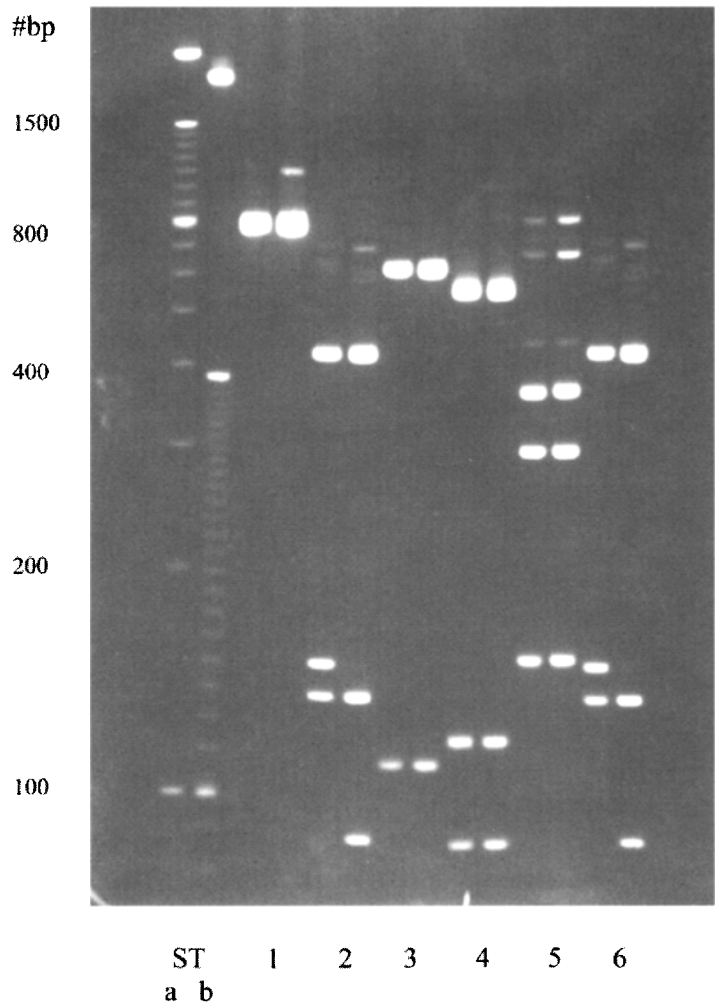

Figure 3. A 6% polyacrylamide gel showing Robust and Stander DNA fragments that were amplified with STS primers 3 and 4 and digested with various restriction endonucleases that have 4-base recognition sites. The lane marked ST(*a*) is a 100-bp ladder DNA size standard, while ST(*b*) is a 10-bp ladder DNA size standard. The remaining lanes were loaded with paired digests of DNA from Robust (left) and Stander (right). Lane pair 1 contained the uncut 800-bp fragments from each variety. The DNA loaded onto lane pair 2 was cut with the restriction enzyme *Alu* I; lane pair 3, *Hae* III; lane pair 4, *Hha* I; lane pair 5, *Hinf* I, and lane pair 6, a repeat with *Alu* I. Even though all restriction enzymes cut the 800-bp STS fragment, only *Alu* I generated a polymorphism. This is consistent with the DNA sequence given in Table 3.

was unique in that it cloned and size-selected a mixed RAPD amplified fragment rather than manually isolating and then cloning a single fragment from a gel. This procedure may also be applied to other plant species and situations, such as gene tagging, in which it would desirable to convert an RAPD marker to an STS marker.

Table 3. Partial sequences of DNA from Stander templates of a fragment of approximately 800 bp that was generated by STS primers 3 and 4. The nucleotides in bold indicate putative SNPs between the 2 varieties. The substitutions found in Robust DNA are listed in parentheses. The underlined sequences indicate *Alu* I restriction sites, of which Stander has two and Robust has one because of the presence of a G-A substitution between Stander and Robust. The dotted lines indicate unreadable portions of the 800-bp DNA fragment.

5'

....TCAGCGGCCCTGGTCGTGCATCCCATCATCATTGATGGTATCATCACTAGATCCTCATGGATGGA

GGTATCATCCTCAATCTGGTTTACGAAGACACGGTGAGAAAAATGGGTATCGATCCGTGAAGGAT

CAATCCTCGAGGCTCGCTTGCTCGGGCACTATAACTTTGGAAGCAGTGTTCTGTTCGCCGACAAC

TTCTGAAGCGAAGACTTGATCTTGACATTGCTCTTCACACCGTAT.....TTTAATG GCGTCCCC

(G) (C)

*Alu*I

ATTGCATACCTGAAGCTGAAGACAAGGACTCAATGGTGTTATTACCGCGAATGGTAATACCGATG

(G) (T)

TTATTACCGCGAATGGTAATACCGAGCGGTCCCTCCGAACCATGGAACTTACTGTGGCTCTGGCA

*Alu*I

CAGGCAGCTGAAGTACACCCCAACAATACAACTCCAAAGCACCAGCATGTTAAGGAACACAAATG

(C)

ACGTCTCGCGCTTTGCCATAGTTCTGGATCCTTCTCACGCTCAAACTAAATAGGGATATGCTCCC

3'

CACAACAAGCCCTCGC.....

Acknowledgments

The authors thank the American Malting Barley Association for supporting this research. The technical assistance of Jill McNeil, Irene Shackelford, and Charlotte Burton is also appreciated.

References

Chee PW, Matejowski A, Kanazin V, and Blake T (1993) Development of polymerase chain reaction for barley genome analysis. J Am Soc Brew Chem 51: 93-96.

Dahleen LS (1997) Mapped clone sequences detecting differences among 28 North American barley cultivars. Crop Sci 37: 952-957.

Doyle JJ and Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19: 11-15.

Gebre H, Khan K, and Foster AE (1986) Barley cultivar identification by polyacrylamide gel electrophoresis of hordein proteins. Crop Sci 26: 454-460.

Habernicht DK and Blake TK (1999) Application of PCR to detect varietal purity in malt. Am Soc Brew Chem 57: 64-71.

Heisel SE, Peterson DM, and Jones BL (1986) Identification of United States barley cultivars by sodium dodecyl sulfate poly-acrylamide gel electrophoresis of hordeins. Cereal Chem 63: 500-505.

Hoffman DL and Goates BJ (1990) Polymorphism and genetic basis of β-N-acetylglucosaminidase, hexokinase, and basic β-D-galactosidase in barley (*Hordeum* spp.). Biochem Genet 28: 487-493.

Hoffman DL and Bregitzer P (1996) Identification of reproducible PCR-RAPD markers that enable the differentiation of closely related six-rowed malting barley (*Hordeum vulgare* L.) cultivars. J Am Soc Brew Chem 54: 172-176.

Jones BL and Heisel S (1991) Differentiation of U.S. malting varieties by electrophoresis of esterase isozymes. J Am Soc Brew Chem 49: 93-98.

Penner GA, Bush A, Wise R, Kim W, Domier L, Kasha K, Laroche A, Scoles G, Molnar SJ, and Fedak G (1993) Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. PCR Meth and Appl 2: 341-345.

Saghai Maroof MA, Zhang Q, and Chojecki J (1994) RFLPs in cultivated barley and their application in the evaluation of malting quality cultivars. Hereditas 121: 21-29.

Tragoonrung S, Kanazin V, Hayes PM, and Blake TK (1992) STS facilitated PCR for barley genome mapping. Theor Appl Genet 84: 1002-1008.

Welsh J and McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucl Acids Res 18: 7213-7218.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, and Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl Acids Res 18: 6531-6535.